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SHORT COMMUNICATION

Recovery of Recombinant Viruses in Transgenic Plants

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The 3′ untranslated region (UTR) adjacent to the capsid gene is frequently included with the transgene in the construction of capsid protein mediated virus resistant transgenic plants. Since ribonucleotide sequences within the 3′ UTR are involved in the initiation of viral replication, the presence of this sequence may encourage the participation of the transgene in RNA recombination. Experiments were designed to explore the involvement of the 3′ UTR of cowpea chlorotic mottle virus (CCMV) in RNA recombination events between transgenes and challenging viruses. *Nicotiana benthamiana* was transformed with CCMV transgenes consisting of the 3′ two-thirds of the capsid gene and fragments of the associated 3′ UTR lacking the terminal 69, 83, or 214 nucleotides. Plants were inoculated with wild-type CCMV transcripts for RNAs 1 and 2 and a movement defective RNA 3 transcript lacking the 3′ third of the capsid gene. While no recombinant virus was detected in plants expressing 3′ deletion constructs, 3% of control transformants containing an identical segment of the capsid gene with the complete 3′ UTR yielded viable recombinant virus. These results suggest that RNA recombination between transgenic RNA and viral RNA can be reduced significantly by omitting or disrupting the 3′ UTR in the transgene.

Virus resistant transgenic plants (VRTPs) acquire pathogen derived resistance through the constitutive expression of a segment of a plant virus genome including the capsid (*1*), polymerase (*2*), or movement protein genes (*3, 4*). One concern about the release of VRTPs to the environment is that recombination between the viral transgene and a challenging virus could produce chimeric viruses with distinct properties.

RNA recombination appears to be a fundamental evolutionary mechanism of RNA viruses. Recombination occurs when the viral replicase switches RNA templates during synthesis of the complementary RNA strand and effectually unites two previously distinct RNAs (*5*). Sequence analysis provides evidence of recombination in several animal RNA viruses, bacteriophage (*5*), and plant RNA viruses (*6, 7*). Additionally, RNA recombination has been demonstrated during reverse transcription between a cauliflower mosaic virus (CaMV) transgene and challenging CaMV inoculum (*8, 9*). Both homologous recombination and aberrant homologous recombination have been recorded in bromoviruses (*10–12*). In bromoviruses, nonhomologous recombination is facilitated by heteroduplex formation during minus strand synthesis (*13*).

Cowpea chlorotic mottle bromovirus (CCMV) consists of two monocistronic RNAs, 1 and 2, that encode replication proteins and a dicistronic RNA 3. RNA 3 encodes the putative movement protein, 3a, and capsid protein, both of which are required for systemic movement (*10*). Infectious transcripts produced from complementary DNA (cDNA) clones of these RNAs (*14*) infect legumes systemically and produce a symptomless systemic infection in *Nicotiana benthamiana* (Domin).

Using CCMV as a model system, we established that viral RNA transcribed in a transgenic plant is available for recombination with challenging viruses (*15*). When transgenic *N. benthamiana* expressing the 3′ two-thirds of the capsid gene and the complete 3′ untranslated region (UTR) of CCMV was inoculated with a CCMV systemic movement defective mutant lacking the 3′ third of the capsid gene, 3% of the transformants became systemically infected. Viable recombinant virus with a restored capsid gene was recovered in each case.

Inclusion of the 3′ UTR in the transgenic message may influence the interaction between the transgenic RNA and challenging viral RNA. Given the critical role of the 3′ UTR as the viral replicase binding site in bromoviruses (*16, 17*) and other RNA viruses (*18–20*), this study was undertaken to determine if disruption of the 3′ UTR of a CCMV transgene would influence the formation of viable recombinant viruses.

The three wild-type (WT) CCMV full-length cDNA clones

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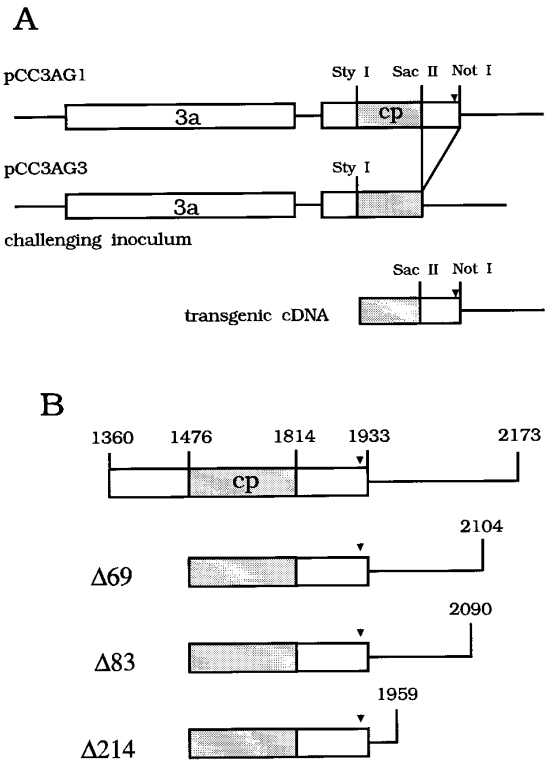

FIG. 1. Representation of constructs derived from pCC3AG1. (A) Full-length CCMV RNA 3 cDNA clone pCC3AG1 is the parent clone for the inoculum and transformation constructions. Genes are indicated by open boxes and UTRs by solid lines. The *Not*I site and a silent mutation, denoted by the arrow, distinguish pCC3AG1 from wild-type CCMV RNA 3. Appropriate restriction sites within the capsid gene, cp, are indicated. RNA 3 inoculum transcribed from pCC3AG3 contains a deletion within the cp gene between *Sac*II (1814–1815) and *Not*I (1933–1934). Plants expressing transgenic cDNA with a complete 3′ UTR served as a control. (B) The complete CCMV cp gene and 3′ UTR are represented on the first line; numerals indicate specific nucleotides. The deletion series with the abbreviated 3′ UTRs are shown below. Numbers prefixed by delta (Δ) indicate the extent of the 3′ deletion. Gray areas indicate sequence shared by the transgenic insert and challenging inoculum.

and their corresponding infectious transcripts, designated C1, C2, and C3, were described previously (*14*). C1 and C2 were not modified for these experiments. Infectious clone pCC3AG1 (Fig. 1A) contained a complete cDNA copy of CCMV RNA 3 adjacent to a T7 RNA polymerase promoter but differed from wild-type C3 by three introduced mutations (*15*). The RNA 3 deletion inoculum, AG3, which lacked the 3′ one-third of the capsid gene, was transcribed from pCC3AG3 (Fig. 1A) as described (*15*).

To generate the 3′ UTR deletions, 1 μg of pCC3AG1 was cut within the polylinker with *Pst*I and *Xba*I and digested at 30° with 3 units exonuclease III. Ten equal aliquots were taken at 5-sec intervals. Plasmid termini were filled with Klenow and plasmids were religated. Enzymatic reactions followed published protocols (*21*) and were conducted under conditions specified by the supplier (Boehringer-Mannheim, Indianapolis, IN).

Following transformation and propagation in *Escherichia coli* strain JM101, plasmids were isolated by alkaline lysis (*22*). Sequence analysis (Sequenase 2.0, U.S. Biochemical, Cleveland OH) revealed a series of nested deletions which removed from 20 to 250 nucleotides of the 3′ UTR. Analysis of the remainder of the 3′ UTRs and capsid genes indicated that no spurious mutations had been introduced and that the marker mutations that distinguish pCC3AG1 from wild-type CCMV RNA 3 remained intact. Three plasmids containing deletions of 69 (pCC3AG2Δ69), 83 (pCC3AG2Δ83), and 214 (pCC3AG-2Δ214) nucleotides from the 3′ terminus of the cDNA insert (Fig. 1B) were selected

Restriction fragments produced by a *Sty*I–*Hin*dIII digest, cDNA nucleotides 1476–1477 and polylinker cleavage site, respectively, of the 3′ deletion plasmids included the 3′ two-thirds of the capsid gene and the remaining 3′ UTR. Binary transformation vector pGA643 (*23*) was digested with *Hin*dIII and treated with alkaline phosphatase. In a two-step ligation process, the *Hin*dIII end of the restriction fragment was ligated to the plasmid. Incompatible sticky ends of the plasmid and insert were filled with Klenow and blunt end ligated. This placed the CCMV sequence between the 35S CaMV transcription promoter and the termination sequences of the octopine-type Ti plasmid pTi63 (*23*). The resulting plasmids, pGACCMVΔ69, pGACCMVΔ83, and pGACCMVΔ214, were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (*24*) by triparental mating using helper plasmid pRK2013 (*25*). *N. benthamiana* leaf explants were transformed, selected, and regenerated as described (*15*).

Transformation was confirmed by enzyme-linked immunosorbant assay (ELISA) for NPTII (5 Prime → 3 Prime, Inc. Boulder, CO) and by Northern blot hybridization with a nick-translated (Boehringer Mannheim) 456-basepair *Sty*I–*Not*I fragment from pCC3AG1. From 30 transformed plants, 1 independent transformant for each deletion was selected for further study and propagated clonally by either tissue culture or cuttings. Selected transformants had NPTII ELISA absorbance values and transcript hybridization signals (Fig. 2) that were similar to those of the control transformants and the transformants used in previous recombination experiments (*15*). Since a full-length capsid protein was not expressed, all transformants were susceptible to WT CCMV infection.

Transgenic plants were inoculated at the 5-leaf stage of development with C1, C2, and AG3. Control plants that transcribe the 3′ two-thirds of the capsid gene and a full-length 3′ UTR (Fig. 1A) were inoculated similarly. Since CCMV is symptomless on *N. benthamiana,* systemic infections of noninoculated leaves were assessed by dot blot assays with RA518, a probe specific for the 3′ UTR of CCMV RNAs (*10*). Hybridization assays indicated that of 156 (Δ69), 172 (Δ83), and 151 (Δ214) transgenic plants inoculated with C1, C2, and movement defective AG3, none became systemically infected over the 4-month screening period. In contrast, 3% (7 of 235) of

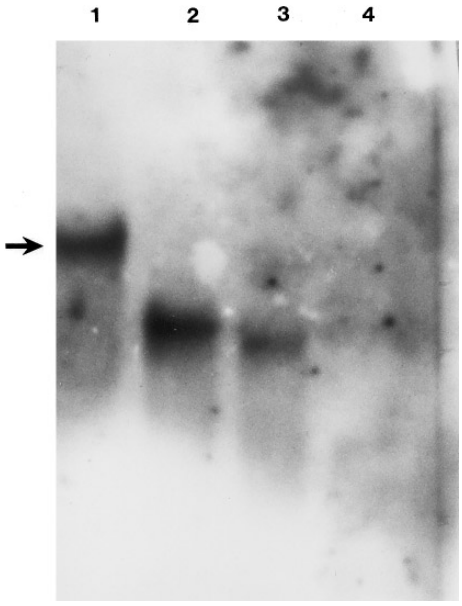

FIG. 2. Northern blots of total RNA of transgenic plants. Equal quantities of total RNA extracted from transgenic plants were separated in a 1.0% denaturing agarose gel, Northern blotted to nylon membrane, and probed with the *Sty*I–*Not*I CCMV capsid gene fragment. Lanes 1–4 contain total RNA from the following transformants: control plant 3-57 with the complete 3′ UTR, Δ69, Δ83, Δ214. The 697 ribonucleotide transcript of 3-57 is denoted by the arrow.

transformants expressing the 3′ two-thirds of the capsid gene plus the full-length 3′ UTR became systemically infected between 2 and 5 weeks postinoculation when challenged with the same inoculum. Recovery of transgenic marker mutations confirmed the recombinant origin of these systemic infections (data not shown). All nontransgenic plants inoculated with wild-type transcripts became systemically infected while plants inoculated with buffer remained uninfected. None of the infected plants showed symptoms which is consistent with wild-type CCMV infection of *N. benthamiana.* Collectively, these data suggested that RNA recombination had either not occurred in the transformants with 3′ UTR deletions or recombination did not result in the restoration of viable virus.

Although numerous reported VRTPs include the 3′ UTR, this region may not be required for resistance since it is not a characteristic of all successful resistance constructs (*26, 27*). While it may provide stability to the transcript, we identified 3′ deletion transformants with transcript quantities similar to those found in transformants expressing the full-length 3′ UTR (Fig. 2). Therefore, a complete UTR may be unnecessary for either transcript stabilization or resistance.

The lack of recombination in these experiments suggests that the 3′ UTR plays a significant role in RNA recombination in transgenic plants. The presence of the complete 3′ UTR on transgenic transcripts may enable a viral replicase complex to initiate replication on the transcript itself, in addition to the viral RNA. This may increase the incidence of RNA recombination in two ways:

First, initiation of replication on the transgenic transcript could result in the synthesis of a complete minus strand RNA complementary to the transgenic viral transcript. In this case, both plus and the minus strand copies of the transgene would be available for RNA recombination.

Second, if replication is initiated on the 3′ UTR of a transgenic transcript, only one template switch would be required to regenerate a complete viral RNA (Fig. 3A). In contrast, if replication cannot initiate on the transgenic insert, a minimum of two template switches is required to restore complete viral RNA. In this case the replicase complex must initiate synthesis on the challenging viral RNA, switch to the viral transcript, and return to a viral RNA to complete synthesis of a full-length viral RNA with complete 3′ and 5′ termini (Fig. 3B). Thus the elimination of the replication initiation site from the transgenic transcript may complicate the formation of viable recombinants significantly.

Assuming that replication initiates only on the challenging inoculum, an alternative explanation is that the shorter transgenic transcripts simply provide less RNA for crossover to occur. However, in this case, we would predict a correlation between the size of the deletion and the recovery of recombinant virus.

In closely related brome mosaic bromovirus, the 3′ UTR of RNA 3 interacts with tRNA specific host enzymes (*28*) and may be involved with the recruitment of host factors required for active replication. If the 3′ UTR of CCMV plays a similar role, this region may target the

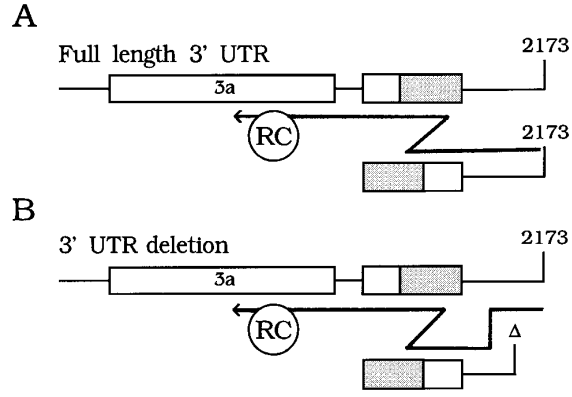

FIG. 3. Models for RNA recombination. CCMV RNA 3 deletion mutant is depicted undergoing recombination with the transgene. In A, the replication complex, RC, has initiated minus strand synthesis, dark line, on the transgene with the full-length 3′ UTR. One strand switch within the shaded area to the inoculum permits synthesis of a full-length RNA 3 to be completed on the deletion mutant. In B, the deletion, Δ, in the transgene disrupts the replication initiation site. Synthesis of a full-length RNA requires initiation on the deletion inoculum, a switch to the transgene and a second switch to the inoculum for completion of minus strand.

transcript to the replication complex, thus increasing the possibility of interactions between the challenging virus and the transgene.

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